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Abstract: The acute-phase response is an inflammatory process triggered mainly by the cytokine IL-6. Signaling of IL-6 is transduced by activation of STAT3 (signal transducer and activator of transcription 3), which rapidly induces the production of acute-phase proteins such as haptoglobin and fibrinogen. Another target of the IL-6/STAT3 signal transduction pathway is the microRNA cluster miR-17/92. Here, we investigated the interplay of miR-17/92 and STAT3 signaling and its impact on the acute-phase response in primary human hepatocytes and hepatoma (HepG2) cells. Employing a reporter gene system consisting of STAT3-sensitive promoter sequences, we show that the miR-17/92 cluster member miR-18a enhanced the transcriptional activity of STAT3. IL-6 stimulation experiments in miR-18a-overexpressing hepatocytes and HepG2 cells revealed an augmented acute-phase response indicated by increased expression and secretion of haptoglobin and fibrinogen. This effect was due, at least in part, to repression of PIAS3 (protein inhibitor of activated STAT, 3), a repressor of STAT3 activity, which we identified as a novel direct target of miR-18a. Finally, we demonstrate that the expression of miR-17/92 in primary hepatocytes and HepG2 cells is modulated by IL-6. Our data reveal, for the first time, a microRNA-mediated positive feedback loop of IL-6 signal transduction leading to an enhanced acute-phase response in human hepatocytes.

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MicroRNA-18a enhances the IL-6 mediated production of the acute-phase proteins fibrinogen and haptoglobin in human hepatocytes

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Acute-phase response is an inflammatory process mainly triggered by the cytokine interleukin-6 (IL-6). Signaling of IL-6 is transduced by the activation of the signal transducer and activator of transcription (STAT)3, that rapidly induces the production of acute-phase proteins such as haptoglobin (Hp) and fibrinogen. Another target of the IL-6-STAT3 signal transduction pathway is the microRNA (miRNA) cluster miR-17/92. Here, we investigated the interplay of miR-17/92 and STAT3 signaling and its impact on the acute-phase response in primary human hepatocytes and hepatoma (HepG2) cells. Employing a reporter gene system consisting of STAT3 sensitive promoter sequences, we show that the miR-17/92 cluster member miR-18a enhanced the transcriptional activity of STAT3. IL-6 stimulation experiments in miR-18a over expressing hepatocytes and HepG2 cells revealed an augmented acute-phase response indicated by increased expression and secretion of Hp and fibrinogen. This effect was, at least in part, due to the repression of the protein inhibitor of activated STAT 3 (PIAS3), a repressor of STAT3 activity, which we identified as a novel direct target of miR-18a. Finally, we demonstrate that the expression of miR-17/92 in primary hepatocytes and HepG2 cells is modulated by IL-6. Our data reveal for the first time a miRNA mediated positive feedback loop of IL-6 signal transduction leading to an enhanced acute-phase response in human hepatocytes.

Severe inflammation is observed in several conditions including overwhelming infection, systemic-inflammatory response syndrome (SIRS), sepsis, and septic shock and results in the activation of a signaling cascade that is commonly referred to as acute-phase response. Acute-phase proteins are involved in a great variety of physiological and biochemical processes including growth inhibition of microbes (e. g. complement factors), blood coagulation (e. g. fibrinogen), and binding of proteins (e. g. haptoglobin-mediated binding of hemoglobin) (1). During inflammation, the production of plasma proteins by hepatocytes is altered, either by increasing the levels of plasma proteins (positive acute-phase reaction) or by decreasing their levels (negative acute-phase reaction).

Interleukin-6 (IL-6) is one of the most important cytokines that orchestrates the hepatic production of acute-phase proteins (2). For signal transduction, IL-6 binds to a membrane associated receptor complex, which subsequently leads to the phosphorylation of tyrosine residue 705 (Tyr705) of the signal transducer and activator of transcription (STAT)3 (3). Phosphorylated STAT3 is actively transported to the nucleus where it activates the transcription of IL-6 target genes such as the acute-phase genes haptoglobin (Hp) (4) and fibrinogen gamma chain (FGG) (5). Several negative feedback loops antagonize the activation of STAT3 to avoid persistent downstream signaling and subsequent dysregulation in the production of acute-phase proteins. A well characterized feedback pathway includes the blocking of the phosphorylation of STAT proteins by the action of the cytokine inducible family of suppressors of cytokine signaling (SOCS) (6).

MicroRNAs (miRNAs) comprise a novel class of short non-protein-coding RNAs that regulate the expression of their target genes in a post-

transcriptional manner either by mRNA degradation or by translational repression (7). The importance of miRNAs in the signal transduction of IL-6 has been highlighted by a recent study showing that miRNAs derived from the miRNA cluster miR-17/92 modulate STAT3 phosphorylation in multiple myeloma cells (8); moreover, Fort and co-workers identified several miRNAs that are directly involved in the production of the acute-phase protein fibrinogen in human hepatoma (HuH7) cells (9). The polycistronic miRNA cluster miR-17/92 codes for six mature miRNAs (miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92) and has primarily attracted attention for being frequently linked to cancer by exhibiting oncogenic activities mainly due to targeting tumor suppressor genes (10). The expression of miR-17/92 is regulated by a number of known transcription factors including the oncogenic transcription factor c-Myc (11). In a previous work we provided evidence that, upon stimulation with IL-6, the expression of miR-17/92 is directly regulated by STAT3 thus emphasizing a potential link between miR-17/92 and inflammatory processes (12). Based on these findings we hypothesized that miR-17/92 might represent a key player in IL-6 signal transduction contributing to the regulation of the acute-phase response in hepatocytes.

Since hepatocellular carcinoma (HepG2) cells have been extensively used to investigate the IL-6 induced expression of acute-phase proteins and, thus, provide an established model to study the acute-phase response (13), we used these cells in the present study to address the role of miR-17/92 for expression of acute-phase genes and to identify novel miR-17/92 targets in the IL-6 signaling cascade. In addition, the interaction between miR-17/92 and the acute-phase response was investigated in primary human hepatocytes to underscore the role of these miRNAs in a physiological setting. Our data reveal for the first time a miRNA mediated positive feedback loop of the IL-6 signal transduction leading to an enhanced acute-phase response in human hepatocytes.

EXPERIMENTAL PROCEDURES

Methods are described in detail in the online supplement.

Cell culture- Human hepatocellular carcinoma (HepG2) cells and human embryonic kidney (HEK)293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Human primary hepatocytes were purchased from Lonza (Lonza, Verviers, Belgium) and cultured according to the manufacturer's instructions. For stimulation experiments, HepG2 cells and primary hepatocytes were serum starved for 24h and treated with IL-6 as indicated.

Quantitative real time-PCR (qPCR) analysis- Quantification of specific messenger RNA transcripts (mRNA) was performed by SYBR Green qPCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) with normalization to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Mature miRNAs were detected by specific stem-loop primers according to Chen *et al*. (14). Primer sequences are shown in Suppl. Table S1.

Western blot- The following primary antibodies were used: anti – PIAS3, anti – Hp (both from Abcam, Cambridge, UK), anti - STAT3 (R&D Systems), anti – Phospho-STAT3 (Tyr705, Cell Signaling Technology, Danvers MA, USA), and anti – α-tubulin (Sigma, St. Louis, MO, USA). Bands were detected with species-specific secondary antibodies coupled to horseradish peroxidase and quantified with the Alpha Imager software (Alpha Innotech, San Leandro, CA, USA).

Plasmid construction- An 878-bp-fragment of the Hp promoter containing two STAT3 binding sites and 578 bp of the FGG promoter (one STAT3 binding site) were each cloned into the firefly luciferase-based pGL3basic vector (Promega, Dübendorf, Switzerland). The SV40 promoter of pRL-SV40 (Promega) was replaced by the promoter of GAPDH (1063 bp). The 3'untranslated region (UTR) of PIAS3 (924 bp) was cloned into the pGL3control vector (Promega). As negative control, the anti-sense construct was used (15) as well as a construct with the specifically mutated seed match for miR-18a.

Reporter gene assay- For promoter activity studies, HepG2 cells were transfected with pGL3basic-Hp or pGL3basic-FGG, and pRL-GAPDH for normalization. After 24h, the cells were stimulated with IL-6 for 4h. Luciferase activity was measured using the Dual-Luciferase

Reporter Assay System (Promega) and normalized to the activity of *Renilla* luciferase.

For miRNA target validation, HEK293 cells were transfected with pGL3control-PIAS3-3'UTR, pRL-SV40 for normalization, and precursor molecules of miRNAs (pre-miRs). After 24h, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System.

Transfection of small interfering RNAs (siRNAs) and miRNAs- HepG2 cells were transfected with 200nM of siRNAs (Qiagen, Hombrechtikon, Switzerland) or 100nM of pre-miRs or anti-miRs (Ambion/Applied Biosystems; pre-miR-18a, -19a, -20a, -92a or anti-miR-18a) using the cell line nucleofector kit V (Amaxa, Cologne, Germany) or Lipofectamine 2000. Following an incubation period of 48h or 72h, cells were stimulated with IL-6 (20ng/ml).

Enzyme-linked immunosorbent assay (ELISA)- To determine levels of Hp and fibrinogen in cell culture supernatants commercially available ELISA kits were used (GenWay, San Diego, CA, USA) according to the manufacturer's instructions.

Statistics- For statistical analysis, GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used. To compare samples, the paired or unpaired t-test was applied and a p-value < 0.05 was considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All data are shown as mean ± SD or as single raw data (primary hepatocytes).

RESULTS

MiR-17/92 enhances the acute-phase response in HepG2 cells. This study was dedicated to investigating the role of miR-17/92 in IL-6 signaling and acute-phase response. Recently, we have demonstrated that STAT3 directly activates the transcription of miR-17/92 upon IL-6 stimulation in human pulmonary arterial endothelial cells (12). To address the question whether IL-6 enhances the expression of miR-17/92 in HepG2 cells and primary hepatocytes similarly, IL-6 stimulation experiments were performed. As shown in Figure 1A, HepG2 cells were stimulated for 1h with different amounts of IL-6 ranging from 5ng/ml to 100ng/ml, and expression of the primary transcript of miR-17/92 (C13orf25) was assessed as described by O'Donnell *et al*. (11). Treatment with the lowest dose of IL-6 led to a small but significant up regulation of C13orf25 (1.19±0.13 fold, p=0.008, Fig. 1A); the strongest induction of C13orf25 mRNA levels was found when stimulated with 50ng/ml IL-6 (1.54±0.41 fold, p=0.012).

Dose-dependent induction of two acute-phase genes (i.e. Hp and FGG) used as positive controls is shown in Figure 1B and 1C, respectively. These data reflect the potential of IL-6 to efficiently stimulate the production of acute-phase genes in HepG2 cells.

Next, we used primary hepatocytes to address the role of IL-6 and miR-17/92 in another experimental model of the acute-phase response. We thus measured the expression levels of 3 miRNAs derived from miR-17/92 (miR-18a, miR-19a, and miR-20a) 24h after IL-6 treatment, and found that expression level of all these miRNAs was increased in both donors used in this study (Fig. 1D). Together with our previous findings (12) these data underpin a cell-type independent mechanism for the induction of miR-17/92 by IL-6.

In the further study, we investigated the functional consequence of enhanced expression of miR-17/92 in HepG2 cells, in particular the significances for IL-6 signaling and acute-phase response. Therefore, we performed gain-of-function experiments by transfecting HepG2 cells with precursor molecules (pre-miRs) of miR-18a, miR-19a, miR-20a, miR-92a (representing the four functional miRNA families encoded by miR-17/92), and a cocktail of all four. Successful over expression of miRNAs was confirmed by qPCR (Suppl. Fig. S1). The impact of each miRNA on the activity of STAT3 was measured by employing a luciferase-based reporter gene system consisting of STAT3 sensitive promoter sequences of two selected acute-phase genes, i.e. Hp and FGG. In scrambled transfected cells, IL-6 increased the relative Hp promoter activity by 15.48±4.51 fold as compared to unstimulated scrambled transfected cells (Fig. 2A). Interestingly, the increase in promoter activity upon stimulation with IL-6 was significantly more enhanced when HepG2 cells were transfected with miR-18a (26.40±4.47 fold, p=0.002), miR-20a (21.32±3.04 fold, p=0.032), or with a cocktail of all four miRNAs (24.4±5.46 fold, p=0.011). Similar results were obtained when the promoter

activity of FGG in HepG2 cells was analyzed showing a significantly enhanced IL-6 response after transfection of miR-18a, miR-20a, miR-92a, and of the miR-18a, -19a, -20a, -92a cocktail as compared to scrambled transfection (Fig. 2B).

To confirm the results of the reporter gene assay, mRNA levels of both acute-phase genes in HepG2 cells were quantified. IL-6 stimulation for 4h thus induced the expression of Hp by 4.43±1 fold in scrambled transfected cells. Over expression of miR-18a (6.31±0.75 fold, p=0.001), miR-20a (5.92±1.17 fold, p=0.005), miR-92a (5.9±0.6 fold, p=0.005), or the miRNA cocktail (5.57±1.09 fold, p=0.046) significantly enhanced the induction of Hp transcripts (Fig. 2C). Quantification of FGG mRNA levels yielded a similar expression pattern (Fig. 2D). After 4h of stimulation with IL-6, the expression of FGG was induced in miR-18a transfected cells (3.89±1.35 fold, p=0.082) and miR-20a transfected cells (3.64±0.51 fold, p=0.03) when compared to scrambled transfection (2.86±0.93 fold).

Over expression of miR-18a promotes the expression of acute-phase proteins. Since miR-18a and miR-20a showed the most consistent and prominent effects in modulating the acute-phase response on mRNA level, we assessed the secretion of fibrinogen and Hp into the supernatants of HepG2 cells stimulated with IL-6 for 8h or 24h. Fibrinogen release from miR-18a transfected cells collected 8h after stimulation was significantly increased both when left unstimulated (2170±602 ng/ml, p=0.018) as well as in IL-6 stimulated cells (2760±702.6 ng/ml, p<0.001) when compared to scrambled transfection (unstimulated: 1669±312.1 ng/ml; IL-6 stimulated: 2194±556.3 ng/ml; Fig. 3A). Enforced expression of miR-20a did not influence the release of fibrinogen neither in IL-6 naïve cells nor in stimulated conditions. Similarly, after 24h of IL-6 stimulation miR-18a over expressing cells produced significantly more fibrinogen (4899±253.2 ng/ml vs. 4017±173.5 ng/ml in scrambled control, p=0.002; Fig. 3A), whereas transfection of miR-20a failed to enhance the release of fibrinogen.

The same supernatants were assessed for Hp protein release and, again, we found that miR-18a over expression increased the secretion of the acute-phase protein (Fig. 3B). In detail, miR-18a significantly enhanced Hp release both after 8h (166.6±41.46 ng/ml, p=0.02) and after 24h of IL-6 stimulation (420.8±11.43 ng/ml, p<0.001) when compared to scrambled transfected and IL-6 stimulated cells (139.9±32.6 ng/ml and 368.7±5.25 ng/ml, respectively). Hp release in miR-20a over expressing cells remained unaffected. To confirm these findings, identical experiments were performed in primary hepatocytes. As shown in Figure 3C, the IL-6 induced release of fibrinogen after 24h was more pronounced in hepatocytes transfected with precursor molecules of miR-18a as compared to scrambled control. Similarly, we found increased expression of Hp in miR-18a over expressing hepatocytes as indicated by Western blot (Fig. 3D).

The ELISA and Western blot experiments thus confirmed the potential of miR-18a but not of miR-20a to promote expression and release of acute-phase proteins.

Conversely, loss of function experiments by antagonizing the expression of miR-18a led to a decreased acute-phase response in HepG2 cells (Suppl. Fig. S2).

MiR-18a directly targets PIAS3. The previous experiments revealed an enhanced hepatic acute-phase response upon over expression of miR-18a. Since miRNAs have been implicated in the repression of gene expression, we speculated that miR-18a might target a negative regulator of IL-6 signaling. Utilizing a computational screening (TargetScan, Whitehead Institute for Biomedical Research, www.targetscan.org (16)) the 3'UTR of PIAS3 was identified to contain a potential binding site for miR-18a. PIAS3 is endogenously expressed in HepG2 cells and specifically inhibits the DNA-binding activity of STAT3 (17). To investigate whether miR-18a influences PIAS3 expression, we measured mRNA levels of PIAS3 in miR-18a transfected HepG2 cells and primary hepatocytes. As shown in Figure 4A, miR-18a transfection significantly reduced the expression of PIAS3 mRNA in HepG2 cells (by 36±7 %, p<0.001) and in hepatocytes from both donors. Consistent with these data, Western blot experiments showed reduced protein levels of PIAS3 in HepG2 cells transfected with pre-miR-18a (ratio PIAS3/α-tubulin: scrambled transfection: 0.52±0.04, pre-miR-18a transfection: 0.35±0.12, p=0.044, Fig. 4B). To proof specificity of the observed reduction of PIAS3, we additionally quantified PIAS3 protein expression in miR-20a transfected cells. MiR-20a, which was not predicted to target PIAS3, did not change the

protein expression of PIAS3 (ratio PIAS3/α-tubulin: 0.53±0.09, p=0.773, Fig. 4B) implicating an efficient and specific down regulation of PIAS3 by miR-18a.

To address the question whether the observed reduction of PIAS3 expression is directly miR-18a-driven, we performed reporter gene studies in HEK293 cells. The entire 3'UTR of PIAS3 was cloned into the pGL3control vector creating a luciferase reporter system with the respective seed sequence for miR-18a (Fig. 4D). As negative control, the "anti-sense" construct was generated (15). In addition, we mutated the miR-18a seed match sequence in the "sense" construct by introducing four point mutations (3'UTR of PIAS3 ΔmiR-18a, Fig. 4D). Co-transfection of the PIAS3 3'UTR WT construct and pre-miR-18a yielded a significantly reduced relative luciferase activity (0.6±0.07 fold, p<0.001, Fig. 4E). The "anti-sense", as well as the mutated construct, was not affected by over expression of miR-18a. These findings imply a direct interaction between the 3'UTR of PIAS3 and miR-18a.

PIAS3 is an important modulator of the acute-phase response. So far our data demonstrated that miR-18a promotes the acute-phase response and directly inhibits PIAS3, a known repressor of STAT3 activity. Due to the fact that the role of PIAS3 in the acute-phase response was still unknown, and to mimic the effects of miR-18a, we silenced PIAS3 and, as an additional control, STAT3 in HepG2 cells (mRNA levels were reduced by 32±12% and 69±6%, respectively, p<0.001). Next, we performed reporter gene studies to monitor the promoter activity of Hp and FGG in siRNA transfected and IL-6 stimulated HepG2 cells. The IL-6 induced promoter activity of Hp was significantly reduced in STAT3 silenced cells (7.43±2.01 fold, p<0.001) compared to scrambled transfection (21.17±3.55 fold; Fig. 5A). Conversely, the response to IL-6 was increased when the expression of PIAS3 was silenced (28.15±3.63 fold, p=0.015). Promoter activity studies of FGG revealed a similar result showing a significant reduction (silencing of STAT3) and increase (silencing of PIAS3) of the IL-6 response in HepG2 cells, respectively (Fig. 5B). To further support these findings, mRNA levels of Hp and FGG were measured after 4h of IL-6 stimulation. Figure 5C summarizes the quantification of Hp mRNA levels showing an IL-6 induced up regulation of Hp by 7.32±1.41 fold in scrambled transfected cells. Transfection of STAT3 siRNA reduced the IL-6 induced stimulation of Hp mRNA expression (4.78±0.44 fold, p<0.001); in contrast, the expression of Hp was further increased when PIAS3 was silenced concomitantly (9.65±2.15, p=0.015). A similar expression pattern was detected when the same samples were analyzed for the expression of FGG mRNA (Fig. 5D).

MiR-18a promotes phosphorylation and expression of STAT3. The effect of miR-18a on the phosphorylation of STAT3 was tested by performing Western blot experiments. As shown in Fig. 6A, IL-6 treatment induced the phosphorylation of STAT3 and was found to be more pronounced in pre-miR-18a transfected HepG2 cells (ratio STAT3-P/α-tubulin: scrambled transfection: 0.84±0.15, pre-miR-18a transfection: 1.17±0.31, p=0.039, Fig. 6B). Induction peaked 30min after IL-6 stimulation, indicating enhanced activity of STAT3. In addition, the expression of STAT3 was increased on protein levels (ratio STAT3/α-tubulin: scrambled transfection unstimulated: 0.62±0.13, pre-miR-18a transfection unstimulated: 1.12±0.26, p=0.002, Fig. 6C) as well as on mRNA levels (1.68±0.18 fold, p<0.001, Fig. 6D) by pre-miR-18a transfection.

Taken together, our data present a novel IL-6 – miR-17/92 pathway that enhances the expression and release of the acute-phase proteins fibrinogen and haptoglobin by promoting STAT3 phosphorylation and inhibition of PIAS3 thereby augmenting STAT3 activation.

DISCUSSION

In this study we demonstrate a novel regulatory pathway of the acute-phase response. Briefly, we found that IL-6 stimulated the expression of the miRNA cluster miR-17/92 in HepG2 cells and primary hepatocytes representing a novel positive feedback loop of IL-6 signaling through the repression of the STAT3 inhibitor PIAS3. Thus, over expression of the miR-17/92 derived miR-18a enhanced the production of the acute-phase proteins fibrinogen and haptoglobin providing evidence for an important role of miRNAs in the regulation of the inflammatory response.

To a substantial extent, the acute-phase response is controlled by the cytokine IL-6, which is released from monocytes and other immune cells during acute and chronic inflammatory

disorders (18). In the liver, the acute-phase response is initiated by the IL-6 induced phosphorylation and dimerization of the transcription factor STAT3 which subsequently shuttles to the nucleus (3). STAT3 homodimers bind to specific DNA sequences in promoter regions and stimulate the transcription of IL-6 target genes (19). We have recently shown that, in human pulmonary arterial endothelial cells, STAT3 directly activates the transcription of miR-17/92 upon stimulation with IL-6 (12). Here, analogous stimulation experiments with IL-6 in HepG2 cells revealed a dose-dependent up regulation of the primary transcript of miR-17/92 (i.e. C13orf25). Together with our previous results (12) the induction of miR-17/92 by IL-6 thus appears to be a STAT3 mediated and cell-type independent mechanism. In this regard, we identified an evolutionarily conserved core palindromic TT – AA motif with a 4 bp spacing in the promoter of miR-17/92, which is responsible for STAT3 binding (12).

To maintain a physiological steady-state under normal conditions, function and expression of STAT3 need to be finely balanced which is mainly achieved by the concerted action of intracellular feedback loops. We therefore hypothesized that the STAT3 regulated miR-17/92 cluster might be involved in the feedback signaling of STAT3 as well. We thus measured the promoter activity of the STAT3 target genes Hp and FGG in HepG2 cells transfected with miRNA mimics. Over expression of miR-18a and miR-20a resulted in enhanced activation of STAT3 upon IL-6 stimulation as shown by promoter studies. These results were further confirmed by showing increased mRNA expression levels of Hp and FGG upon transfection of miR-18a and miR-20a, respectively. With respect to these findings we conclude that miR-17/92 acts as an enhancer of STAT3 activity and represents a positive feedback loop in the IL-6 signaling cascade. Interestingly, Pichiorri and co-workers demonstrated that another miRNA encoded by miR-17/92, miR-19a, augments STAT3 activity by targeting SOCS1, a known suppressor of STAT3 phosphorylation (8). In contrast to these findings, we did not observe increased IL-6 signaling in miR-19a transfected HepG2 cells. These differences are probably due to the respective experimental settings: since SOCS1 is part of a negative feedback loop of STAT3 activity (6) and, thus, exerts its function with temporal delay, the four hours of IL-6 stimulation employed in the current study might be too short to observe a miR-19a mediated effect on STAT3 activity.

Since miRNAs have mainly been associated with the repression of gene expression (7) the observed up regulation of acute-phase genes upon over expression of miRNAs was most likely due to an indirect effect (i.e. the repression of a yet undefined inhibitor). A computational approach identified PIAS3, which interferes with the DNA binding activity of STAT3 (17), to contain a potential binding site for miR-18a. Over expression of miR-18a decreased the expression of PIAS3 both on mRNA (probably due to mRNA cleavage) and protein level. Furthermore, by performing reporter gene studies comprising the 3'UTR of PIAS3, we confirmed a direct miR-18a driven repression of PIAS3. We demonstrated the importance of PIAS3 for acute-phase response by showing that knockdown of PIAS3 led to enhanced activation of the promoters of FGG and Hp and thus increased expression of Hp and FGG mRNAs. The correlation between stimulation with IL-6 and expression of C13orf25, and PIAS3 was further investigated by kinetical experiments and revealed a down regulation of PIAS3 after long-term stimulation (Suppl. Fig. S3).

To determine whether miR-18a also enhances the secretion of acute-phase proteins and to put our findings into a physiological context, we quantified the levels of Hp and fibrinogen in the supernatants of pre-miRNA transfected HepG2 cells. We found the release of Hp and fibrinogen to be enhanced by miR-18a in both IL-6 naïve and in IL-6 stimulated conditions. These findings emphasize the potency of miR-18a as a regulator of the acute-phase response via STAT3 signaling.
Loss of function experiments by antagonizing miR-18a to blocking STAT3 signaling was performed in HepG2 cells transfected with small anti-sense molecules directed against miR-18a (anti-miR-18a). These experiments showed reduced promoter activity and mRNA expression levels of FGG and Hp as well as decreased secretion of fibrinogen and Hp in anti-miR-18a treated cells. The protein levels of PIAS3, on the other hand, were found to be up regulated under these conditions. These data confirm the pathophysiological role of miR-18a for the IL-6 signaling cascade and further demonstrate that

silencing of miR-18a may provide a useful tool to interfere with the hepatic acute-phase response.

The phosphorylation of tyrosine residue 705 (Tyr705) of STAT3 is an essential trigger of the acute-phase reaction and thus of special importance (3). Here, we found that Tyr705 was more phosphorylated in pre-miR-18a transfected cells indicating enhanced STAT3 activity. These findings support the conclusion that miR-18a acts as enhancer of the IL-6 pathway. Interstingly, the expression of STAT3 by pre-miR-18a transfection was found to be increased on mRNA as well as on protein levels. Since the inhibitory action of PIAS3 affects the DNA binding activity of STAT3, one might propose an additional, PIAS3-independent effect of miR-18a on Tyr705 phosphorylation. Whether this effect is due to increased expression of the unphosphorylated STAT3 substrate remains unclear and needs to be addressed by further studies.

In addition to miR-18a, the STAT3 dependent activation of the Hp and FGG promoters was significantly enhanced when HepG2 cells were transfected with precursor molecules of miR-20a. ELISAs for Hp and fibrinogen, however, did not reveal an elevated release of these proteins when miR-20a was over expressed. Since two independent methods (reporter gene assay and qPCR) showed an increase of STAT3 activity by miR-20a, we suggest that the failure of increased acute-phase protein secretion by miR-20a might be due to an impaired protein release, or, alternatively, that miR-20a targets not only repressors but also activators of the IL-6 transduction pathway, for example the Janus kinase 1 (JAK1). JAK1 is the kinase responsible for STAT3 phosphorylation and is also targeted by miR-20a (20).

The most important findings of this study were confirmed by performing experiments in an additional cell model using primary human hepatocytes. By over expressing of miR-18a in these cells, we could show that miR-18a augments the IL-6 induced acute-phase response by targeting the repressor of IL-6 signaling, PIAS3. Similarly, IL-6 treatment increased the expression of miRNAs derived from the miR-17/92 cluster suggesting the presence of a positive feed back-loop both in primary hepatocytes and in HepG2 cells.

In summary, we propose the following model for the regulation of the acute-phase response by miR-17/92 (Fig. 7): during states of acute or chronic inflammation immune cells secrete IL-6 which causes the activation of STAT3 in hepatocytes. This triggers the production of acute-phase proteins and their systemic release. At the same time the expression of miRNAs derived from the cluster miR-17/92 is up regulated. This leads to a further augmentation of STAT3 activity through the repression of PIAS3 by miR-18a and finally contributes to the overwhelming release of acute-phase proteins.

In conclusion, we identified here a novel IL-6 – miR-17/92 pathway, which, through the inhibition of PIAS3, enhances the production and release of the acute-phase proteins fibrinogen and haptoglobin. To our knowledge this is the first report on a miRNA mediated regulation of PIAS3 thus contributing to the understanding of the complex regulatory mechanisms within the signal transduction of IL-6.

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FOOTNOTES

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Conflict of interest: The authors declare no competing interests.
The abbrevations used are (in order of appearance): IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3; Hp, haptoglobin; miRNA, microRNA; HepG2 cells, hepatocellular carcinoma cells; PIAS3, protein inhibitor of activated STAT 3; SIRS, systemic-inflammatory response syndrome; FGG, fibrinogen gamma chain; SOCS, suppressors of cytokine signaling; HEK293 cells, human embryonic kidney cells; qPCR, Quantitative real time-PCR

FIGURE LEGENDS

Fig. 1. **Dose-dependent induction of C13orf25 and acute-phase genes by IL-6.** HepG2 cells and primary hepatocytes were stimulated with IL-6 for 1h or 24h, respectively. (A) The mRNA levels of C13orf25 were dose-dependently induced by IL-6, with 50ng/ml of IL-6 yielding the strongest increase of C13orf25 (n=7). (B) IL-6 induced the mRNA expression of the acute-phase genes Hp and (C) FGG (n=7). (D) IL-6 stimulation (20ng/ml) for 24h led to increased expression levels of miR-18a, miR-19a, and miR-20a in primary hepatocytes (n=2). The dashed line indicates unstimulated control cells. The respective unstimulated samples were set to 1. Statistical analysis by paired student's t-test.

Fig. 2. **Effect of miR-17/92 on the acute-phase response in HepG2 cells.** HepG2 cells were transfected with pre-miRNA (pre-miR-18a, -19a, -20a, -92a, and a cocktail of all four) or scrambled negative control. (A) Promoter activity of Hp was assessed by co-transfection of a luciferase-based vector system comprising 878 bp of the Hp promoter sequence. 4h of IL-6 stimulation (20ng/ml) induced relative luciferase activity in all samples, while the IL-6 response was more pronounced in miR-18a, miR-20a, and the miRNA cocktail transfected cells (n=5). (B) A luciferase-based reporter gene system consisting of the promoter sequence of FGG (578 bp) was co-transfected in HepG2 cells. The response to

4h of IL-6 stimulation was enhanced by co-transfection of miR-18a, miR-20a, miR-92a, and a cocktail of all as compared to scrambled control (n=7). (C) Quantification of the mRNA levels of Hp after 4h of IL-6 stimulation (20ng/ml) showed a similar result as the respective reporter gene studies. The IL-6 inducible effect on Hp transcription was significantly enhanced by transfection of miR-18a, miR-20a, miR-92a, and the cocktail of all four miRNAs (n=6). (D) Identical samples showing significant alteration of the FGG mRNA expression by IL-6 in miR-20a transfected cells (n = 6). The respective unstimulated samples were set to 1. Statistical analysis by unpaired student's t-test.

Fig. 3. **Over expression of miR-18a promotes the expression and release of acute-phase proteins.** Protein levels of fibrinogen and Hp of pre-miRNA transfected HepG2 cells and hepatocytes were quantified by ELISA or Western blot. (A) Release of fibrinogen into the supernatants after 8h and 24h of IL-6 stimulation (20ng/ml) was significantly increased in cells over expressing miR-18a as shown by ELISA. MiR-20a transfection failed to increase the release of fibrinogen (n≥5). (B) The same supernatants were assessed for quantification of Hp protein levels showing enhanced secretion of Hp after 8h and 24h of IL-6 stimulation when miR-18a was over expressed. Transfection of miR-20a did not change the release of Hp (n≥5). (C) Primary hepatocytes were transfected with pre-miR-18a and stimulated with IL-6 (20ng/ml) for 24h. The total amount of fibrinogen in supernatants was quantified by ELISA showing that the IL-6 induced release of fibrinogen was enhanced in hepatocytes transfected with pre-miR-18a (n=2). (D) The same cells were analyzed for protein levels of haptoglobin using Western blot. The expression of haptoglobin was increased by miR-18a transfection as compared to scrambled transfection (n=2). Statistical analysis by paired student's t-test.

Fig. 4. **PIAS3 is a direct target of miR-18a.** (A) MiR-18a transfected HepG2 cells revealed a significant down regulation of PIAS3 mRNA levels compared to scrambled control (n=7). The mRNA levels of PIAS3 were also found to be decreased by miR-18a transfection in primary hepatocytes (n=2). (B) Western blot confirmed the reduced expression of PIAS3 on protein level in HepG2 cells as assessed by densitometric analysis (n=4). Specific miR-18a-mediated down regulation of PIAS3 was proven by transfection of a non-predicted miRNA, miR-20a, showing no alterations in the protein expression of PIAS3. (C) Western Blots used for densitometric analysis are shown (n=4). (D) TargetScan prediction software identified one seed match of miR-18a in the 3'UTR of PIAS3; the predicted base pairing of PIAS3 mRNA and miR-18a is shown. The entire 3'UTR of PIAS3 was used for reporter gene studies and the predicted miR-18a seed sequence was destroyed by introducing four point mutations. (E) Reporter gene studies in HEK293 cells showed that co-transfection of miR-18a significantly decreased the relative luciferase activity compared to scrambled transfection. The "anti-sense" construct as well as the mutated construct (ΔmiR-18a) were not affected by over expression of miR-18a (n=7). Statistical analysis by paired student's t-test (A, E; scrambled transfection was set to 1) or by unpaired student's t-test (B).

Fig. 5. **PIAS3 is an important mediator of the acute-phase response.** HepG2 cells were transfected with siRNAs targeting PIAS3 (si_PIAS3) and STAT3 (si_STAT3). (A) IL-6 stimulation (20ng/ml) for 4h activated the promoter of Hp in all samples with silencing of STAT3 reducing the activity of luciferase as compared to scrambled transfection. Conversely, silencing of PIAS3 increased the relative luciferase activity (n=5). (B) Promoter studies of FGG revealed a similar pattern of activation showing a significant reduction (si_STAT3) and enhancement (si_PIAS3) of the IL-6 response in HepG2 cells, respectively (n=7). (C) mRNA levels of Hp after 4h of IL-6 stimulation were significantly decreased (si_STAT3) or increased (si_PIAS3) compared to scrambled transfection (n=9). (D) The same samples were analyzed for expression of FGG mRNA expression showing a similar expression pattern (n=9). The respective unstimulated samples were set to 1. Statistical analysis by unpaired student's t-test.

Fig. 6. **MiR-18a promotes phosphorylation and expression of STAT3.** HepG2 cells were transfected with pre-miR-18a or scrambled negative control, serum starved and stimulated with IL-6 (20ng/ml) for 30min, 1h or 4h. (A) Western blot experiments were performed to analyze phosphorylation and expression of STAT3; two representative Western blots are shown. (B) Phosphorylation of STAT3

(Tyr705) was markedly enhanced in pre-miR-18a transfected cells as compared to scrambled transfection. Densitometric analysis revealed a significant increase after 30min of IL-6 stimulation (n=6). (C) Transfection of miR-18a led to a significant up regulation of protein levels of STAT3 in IL-6 naïve as well as in IL-6 stimulated HepG2 cells. Densitometric analysis is shown (n=6). (D) The mRNA levels of STAT3 were found to be up regulated in miR-18a over expressing cells (n=7). Statistical analysis by unpaired student's t-test.

Fig. 7. **Regulation of acute-phase gene expression by miR-17/92 through a positive feedback loop involving PIAS3.** We propose the following model for the regulation of the acute-phase response by the miR-17/92 cluster: IL-6, released from immune cells, activates STAT3 in hepatocytes thus triggering the expression of acute-phase genes and the miRNA cluster miR-17/92. In turn, miR-18a targets the STAT3 inhibitor PIAS3, which leads to the enhanced activation of STAT3 and, thus, results in an increased release of acute-phase proteins.

Figure 1

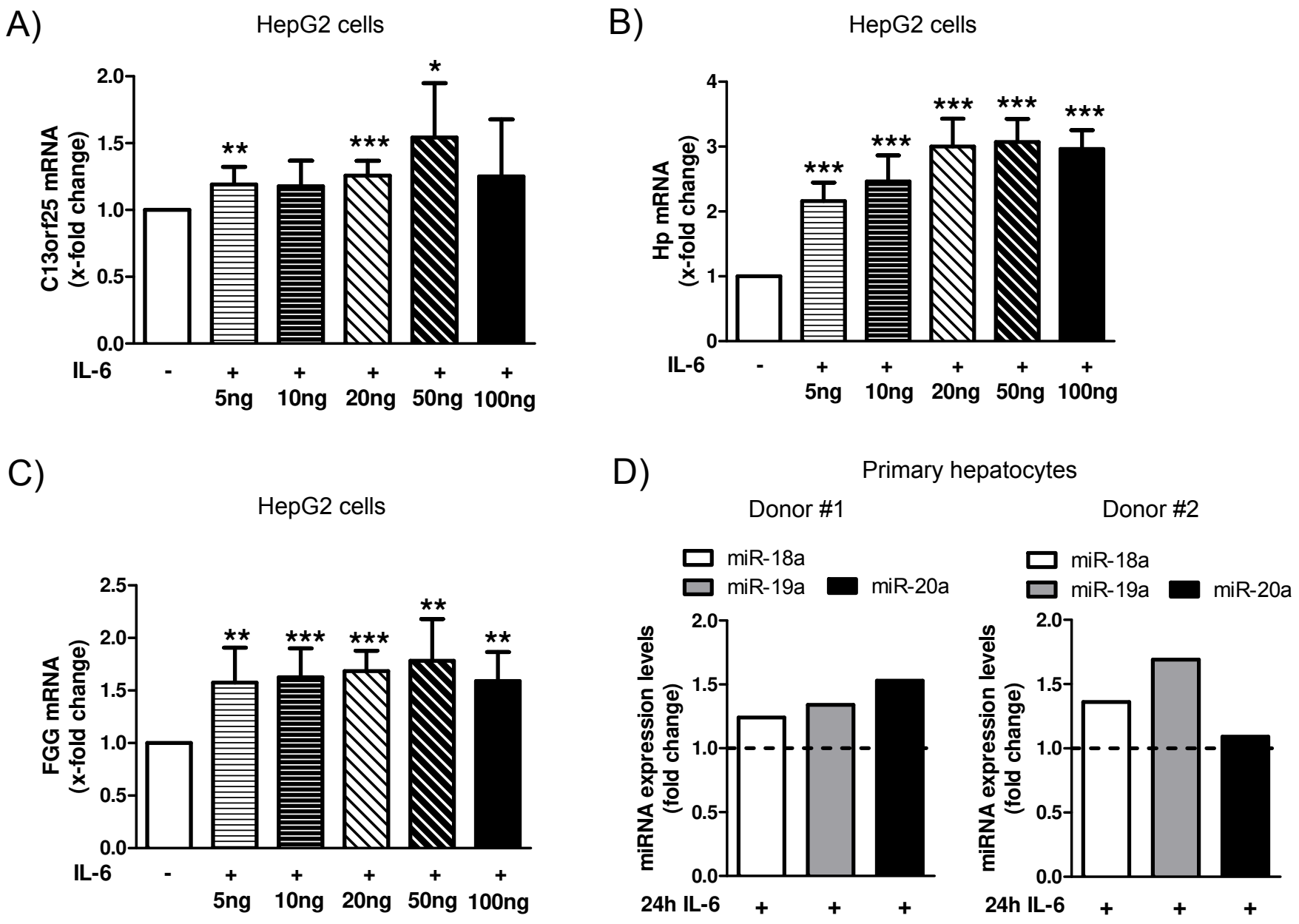
A)
HepG2 cells
C13orf25 mRNA (x-fold change)
2.0
1.5
1.0
0.5
0.0
**
***
*
IL-6 - + + + + +
5ng 10ng 20ng 50ng 100ng
B)
HepG2 cells
Hp mRNA (x-fold change)
4
3
2
1
0
***
***
***
***
***
IL-6 - + + + + +
5ng 10ng 20ng 50ng 100ng
C)
HepG2 cells
FGG mRNA (x-fold change)
2.5
2.0
1.5
1.0
0.5
0.0
**
***
***
**
**
IL-6 - + + + + +
5ng 10ng 20ng 50ng 100ng
D)
Primary hepatocytes
Donor #1
Donor #2
miR-18a
miR-19a
miR-20a
miRNA expression levels (fold change)
2.0
1.5
1.0
0.5
0.0
24h IL-6 + + +

Figure 2

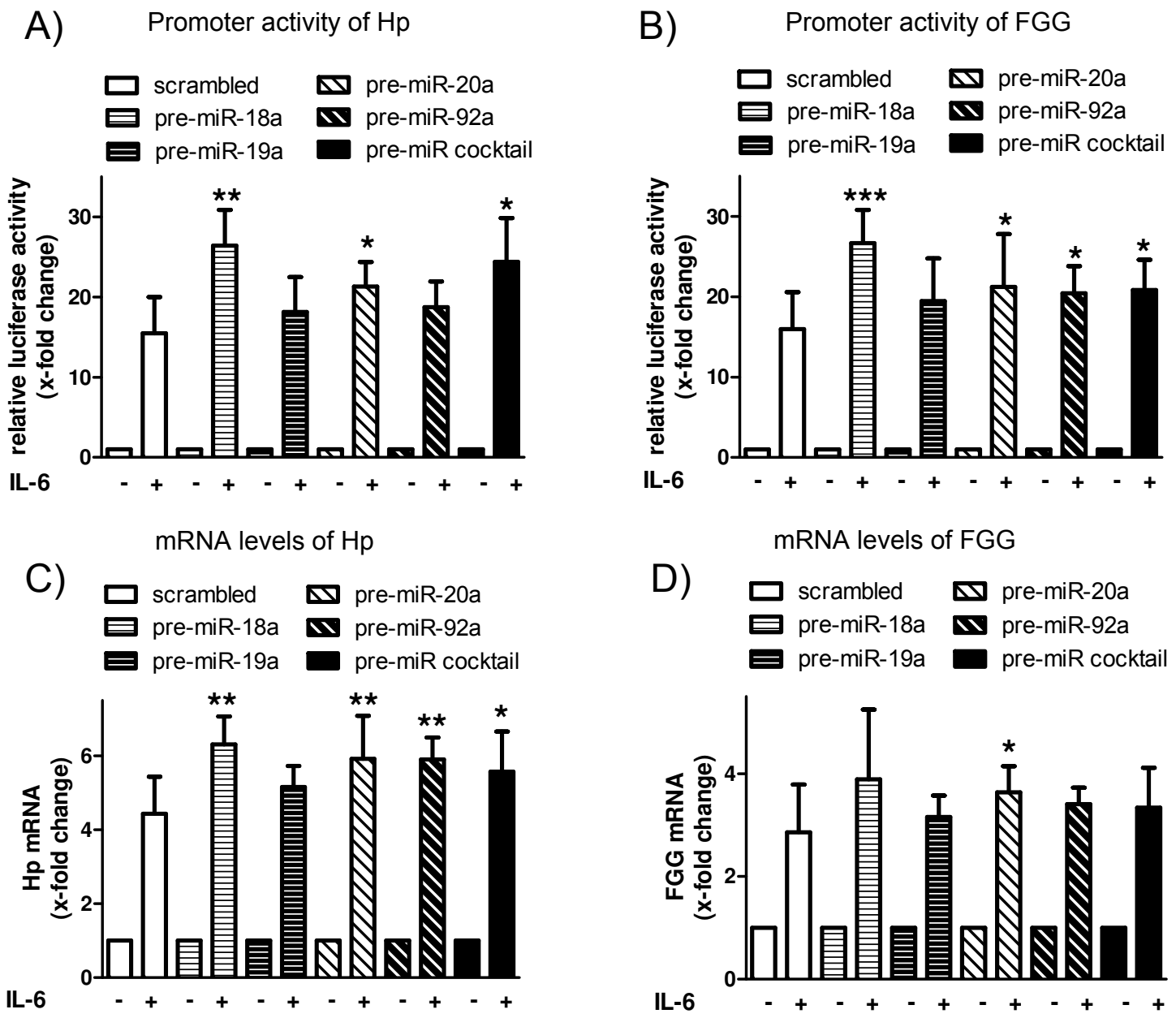
A)
Promoter activity of Hp
scrambled
pre-miR-18a
pre-miR-19a
pre-miR-20a
pre-miR-92a
pre-miR cocktail
relative luciferase activity (x-fold change)
IL-6
B)
Promoter activity of FGG
C)
mRNA levels of Hp
Hp mRNA (x-fold change)
D)
mRNA levels of FGG
FGG mRNA (x-fold change)

Figure 3

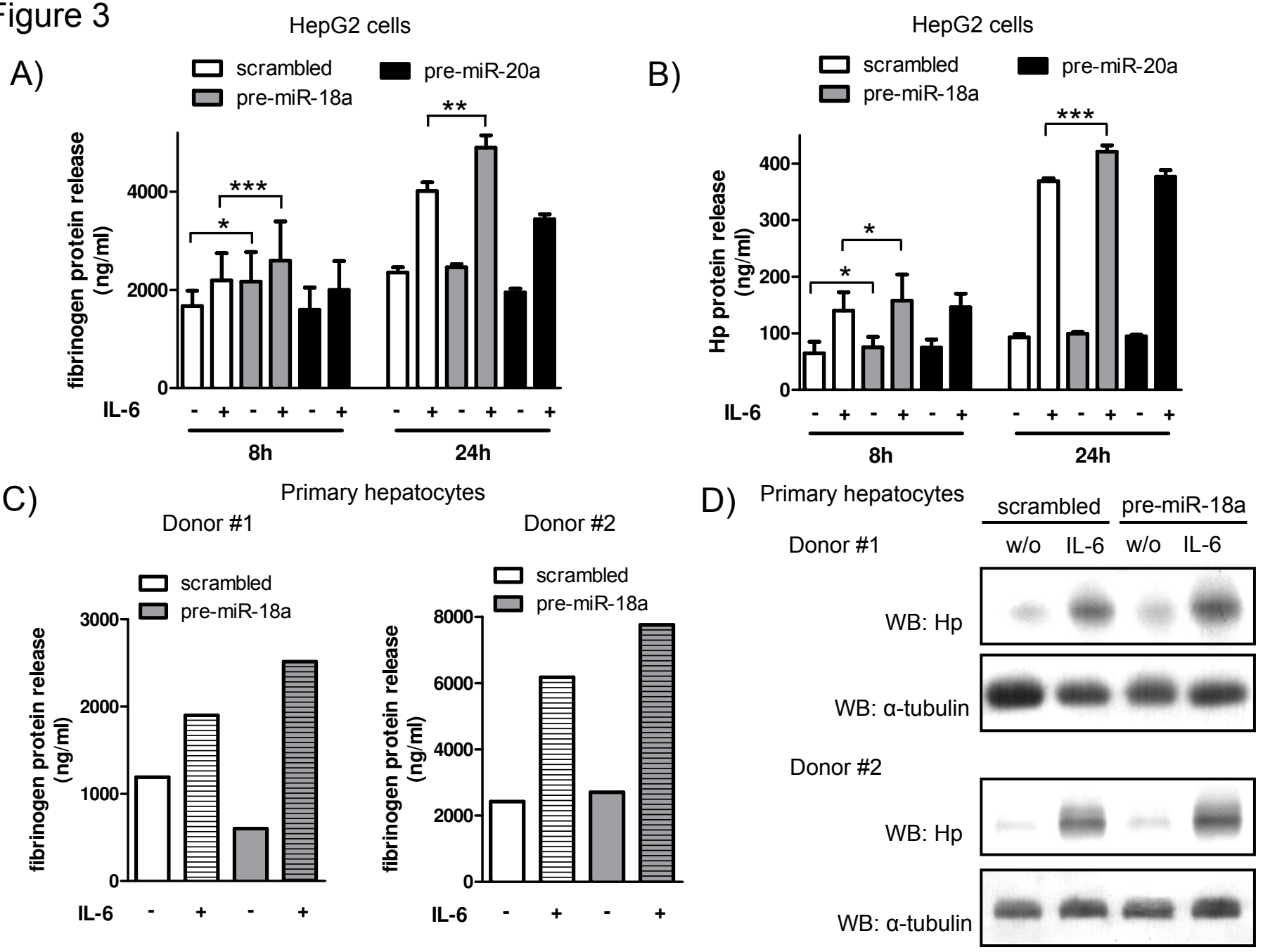
HepG2 cells
A)
scrambled
pre-miR-18a
pre-miR-20a
fibrinogen protein release (ng/ml)
4000
2000
0
*
***
**
IL-6
- + - + - +
8h
24h
HepG2 cells
B)
scrambled
pre-miR-18a
pre-miR-20a
Hp protein release (ng/ml)
400
300
200
100
0
*
*
***
IL-6
- + - + - +
8h
24h
C)
Primary hepatocytes
Donor #1
Donor #2
scrambled
pre-miR-18a
fibrinogen protein release (ng/ml)
3000
2000
1000
0
8000
6000
4000
2000
0
IL-6
- + - +
D)
Primary hepatocytes
scrambled
pre-miR-18a
Donor #1
w/o IL-6 w/o IL-6
WB: Hp
WB: α-tubulin
Donor #2
WB: Hp
WB: α-tubulin

Figure 4

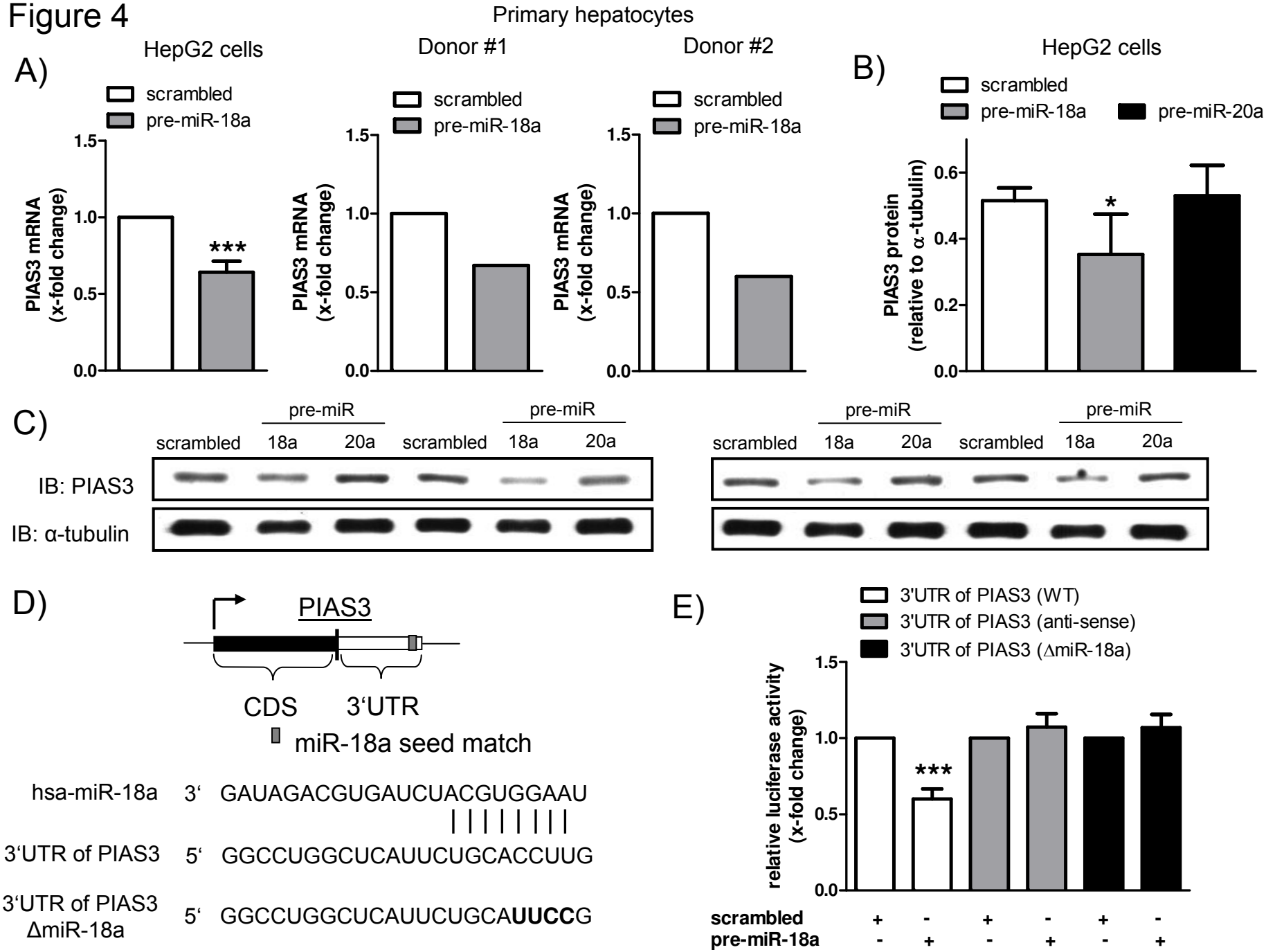
Primary hepatocytes
HepG2 cells
Donor #1
Donor #2
A)
scrambled
pre-miR-18a
PIAS3 mRNA (x-fold change)
1.5
1.0
0.5
0.0
***
B)
HepG2 cells
scrambled
pre-miR-18a
pre-miR-20a
PIAS3 protein (relative to α-tubulin)
0.6
0.4
0.2
0.0
*
C)
pre-miR
scrambled
18a
20a
IB: PIAS3
IB: α-tubulin
D)
PIAS3
CDS
3'UTR
miR-18a seed match
hsa-miR-18a 3' GAUAGACGUGAUCUACGUGGAAU
3'UTR of PIAS3 5' GGCCUGGCUCAUUCUGCACCUUG
3'UTR of PIAS3 ΔmiR-18a 5' GGCCUGGCUCAUUCUGCAUUCCG
E)
3'UTR of PIAS3 (WT)
3'UTR of PIAS3 (anti-sense)
3'UTR of PIAS3 (ΔmiR-18a)
relative luciferase activity (x-fold change)
1.5
1.0
0.5
0.0
***
scrambled + - + - + -
pre-miR-18a - + - + - +

Figure 5

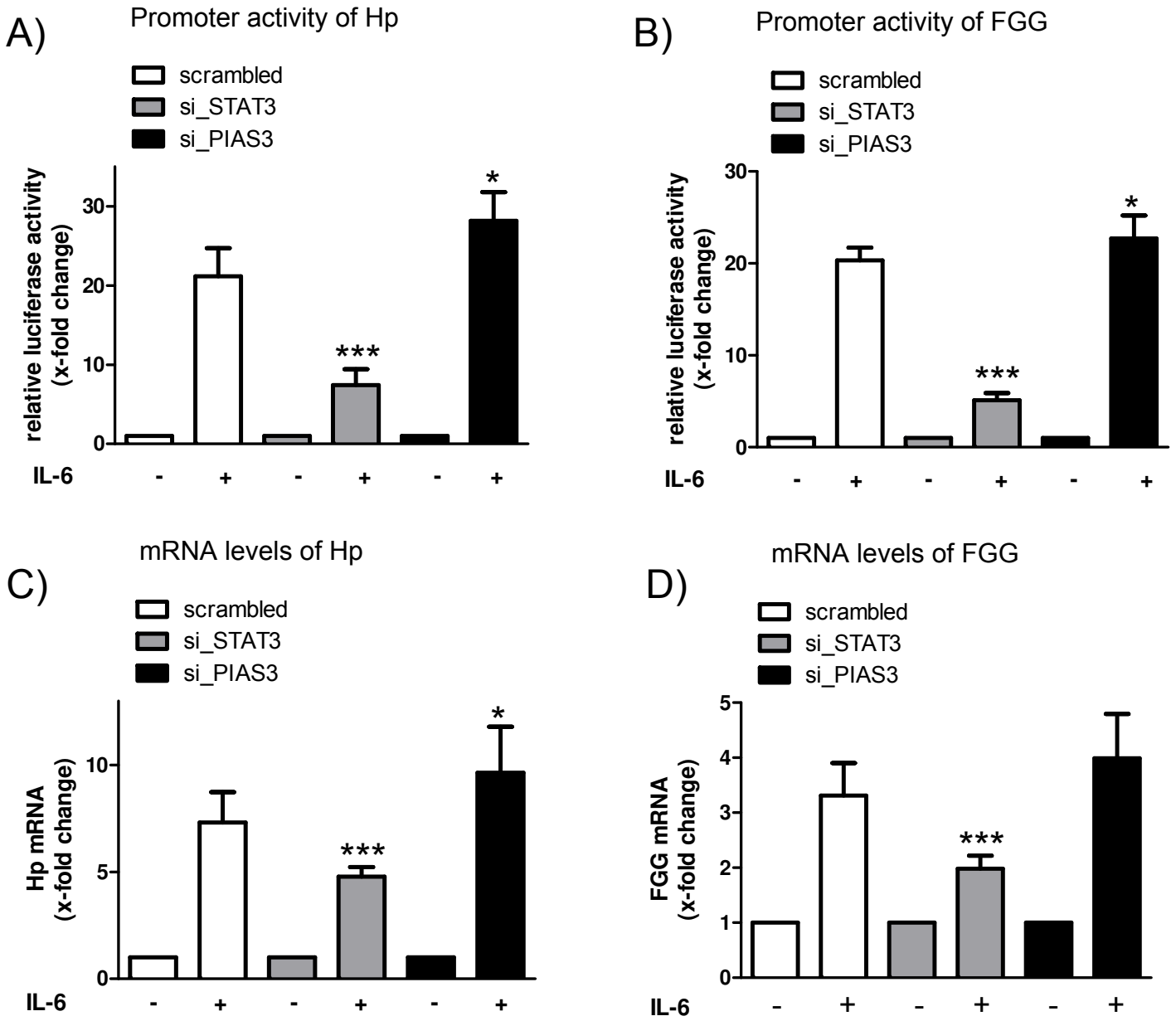
A)
Promoter activity of Hp
scrambled
si_STAT3
si_PIAS3
relative luciferase activity (x-fold change)
30
20
10
0
***
*
IL-6
- + - + - +
B)
Promoter activity of FGG
scrambled
si_STAT3
si_PIAS3
relative luciferase activity (x-fold change)
30
20
10
0
***
*
IL-6
- + - + - +
C)
mRNA levels of Hp
scrambled
si_STAT3
si_PIAS3
Hp mRNA (x-fold change)
10
5
0
***
*
IL-6
- + - + - +
D)
mRNA levels of FGG
scrambled
si_STAT3
si_PIAS3
FGG mRNA (x-fold change)
5
4
3
2
1
0
***
IL-6
- + - + - +

Figure 6

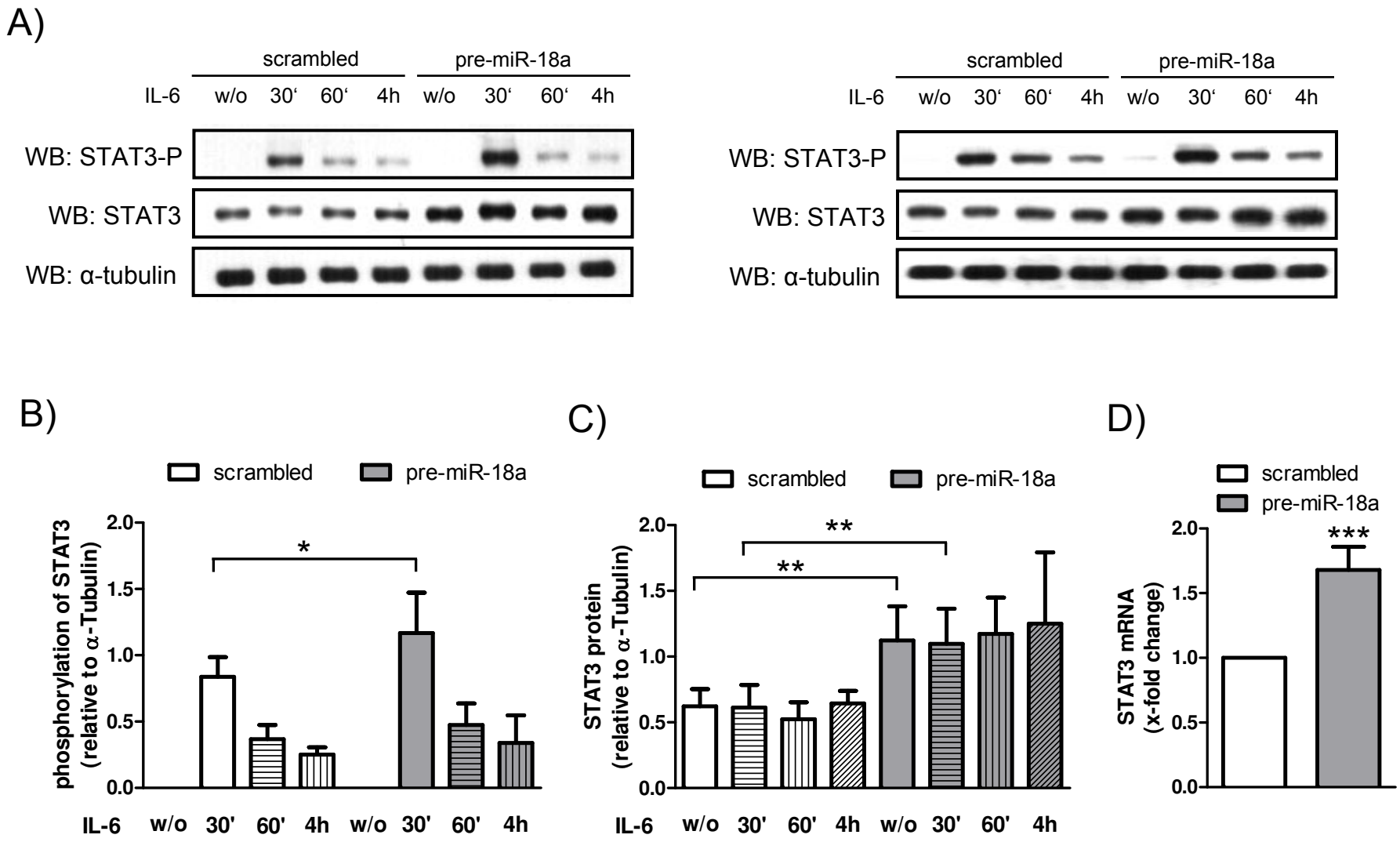
A)
scrambled
pre-miR-18a
IL-6
w/o
30‘
60‘
4h
WB: STAT3-P
WB: STAT3
WB: α-tubulin
B)
scrambled
pre-miR-18a
phosphorylation of STAT3 (relative to α-Tubulin)
2.0
1.5
1.0
0.5
0.0
*
IL-6
w/o
30'
60'
4h
C)
STAT3 protein (relative to α-Tubulin)
**
**
D)
STAT3 mRNA (x-fold change)
***

Figure 7